

# Expression Profiling of Stem Cell-Related Genes in Neoadjuvant-Treated Gastric Cancer: A *NOTCH2*, *GSK3B* and $\beta$ -catenin Gene Signature Predicts Survival

Lukas Bauer<sup>1</sup>, Rupert Langer<sup>1</sup>, Karen Becker<sup>1</sup>, Alexander Hapfelmeier<sup>4</sup>, Katja Ott<sup>2</sup>, Alexander Novotny<sup>3</sup>, Heinz Höfler<sup>1,5</sup>, Gisela Keller<sup>1\*</sup>

**1** Institute of Pathology, Technische Universität München, München, Germany, **2** Department of Surgery, Universität Heidelberg, Heidelberg, Germany, **3** Department of Surgery, Technische Universität München, München, Germany, **4** Institute of Medical Statistics and Epidemiology, Technische Universität München, München, Germany, **5** Institute of Pathology, Helmholtz-Zentrum München, Neuherberg, Germany

## Abstract

Cancer stem cell (CSC) based gene expression signatures are associated with prognosis in various tumour types and CSCs are suggested to be particularly drug resistant. The aim of our study was first, to determine the prognostic significance of CSC-related gene expression in residual tumour cells of neoadjuvant-treated gastric cancer (GC) patients. Second, we wished to examine, whether expression alterations between pre- and post-therapeutic tumour samples exist, consistent with an enrichment of drug resistant tumour cells. The expression of 44 genes was analysed in 63 formalin-fixed, paraffin embedded tumour specimens with partial tumour regression (10–50% residual tumour) after neoadjuvant chemotherapy by quantitative real time PCR low-density arrays. A signature of combined *GSK3B*<sup>high</sup>,  $\beta$ -catenin (*CTNNB1*)<sup>high</sup> and *NOTCH2*<sup>low</sup> expression was strongly correlated with better patient survival ( $p < 0.001$ ). A prognostic relevance of these genes was also found analysing publically available gene expression data. The expression of 9 genes was compared between pre-therapeutic biopsies and post-therapeutic resected specimens. A significant post-therapeutic increase in *NOTCH2*, *LGR5* and *POU5F1* expression was found in tumours with different tumour regression grades. No significant alterations were observed for *GSK3B* and *CTNNB1*. Immunohistochemical analysis demonstrated a chemotherapy-associated increase in the intensity of *NOTCH2* staining, but not in the percentage of *NOTCH2*. Taken together, the *GSK3B*, *CTNNB1* and *NOTCH2* expression signature is a novel, promising prognostic parameter for GC. The results of the differential expression analysis indicate a prominent role for *NOTCH2* and chemotherapy resistance in GC, which seems to be related to an effect of the drugs on *NOTCH2* expression rather than to an enrichment of *NOTCH2* expressing tumour cells.

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\* E-mail: gisela.keller@lrz.tum.de

## Introduction

Advanced gastric carcinomas (GC) are frequently treated by platin/5-fluorouracil (5FU)-based neoadjuvant chemotherapy [1]. The aim of this therapy is, amongst others, to shrink the tumour before surgery to increase the probability of complete resection and to thus improve patient survival. However, response rates are low, and complete or subtotal tumour regression is observed in only 20–40% of the patients [1,2]. Thus, chemotherapy resistance is a major obstacle for successful treatment.

According to the cancer stem cell hypothesis, tumour cells are heterogeneous, and an increased drug resistance is a particular phenotype of a minority of tumour cells – the so-called cancer-initiating cells or cancer stem cells (CSCs) [3–5]. An increase in the CSC population after chemotherapy has been demonstrated [6,7], and stem cell based gene expression signatures were associated with poor prognosis in various tumours including gastric carcinomas [8–11]. The CSC hypothesis is still controversially discussed, but there is evidence for the existence of CSCs in several

tumour types and molecular markers have been identified which are preferentially found on these cells [4,5]. The activation of embryonic signalling pathways, such as the Wnt, Notch and Hedgehog pathways, has been suggested as a driving force for the formation of CSCs [4,12]. Data regarding the source and existence of gastric CSCs remain inconclusive [13–17]. In mice, bone-marrow derived cells or a specific cell population in the antrum expressing the Wnt target molecule *LGR5*, have been associated with CSCs in the stomach [17,18]. In addition, *CD44* and *CD24* have been suggested as specific cell surface markers, but the data are inconsistent [19,20].

The neoadjuvant treatment protocol for GC provides an excellent opportunity to investigate tumour cells before and after chemotherapy in patients. In this study, we aimed to elucidate first, whether the expression of putative CSC-related genes in the post-therapeutic residual tumour predicts patient survival and second, whether particular genes are differentially expressed between pre-therapeutic biopsies and the post-therapeutic tumour specimens, consistent with an enrichment of chemotherapy-resistant tumour

cells as predicted by the CSC concept. The group of patients, who demonstrated considerable tumour shrinkage after neoadjuvant chemotherapy, but still had sufficient residual tumour cells available for analysis (10–50% residual tumour) were considered as the most suitable group to start with a screening analysis for prognostic relevant genes and to then identify relevant differences in gene expression between the pre- and post-therapeutic tumour samples. Analysing these residual tumour cells we identified a gene expression pattern encompassing *GSK3B*, *CTNNB1* and *NOTCH2*, which strongly predicts prognosis of the patients. We show that the impact of *GSK3B* and *CTNNB1* to this signature is not dependent on chemotherapy and more likely reflects a property of the primary tumour and our data further suggest, that in particular *NOTCH2* might play a role for chemotherapy resistance in GC.

## Materials and Methods

### Patients

In total, 480 patients with locally advanced GC (cT3/4) were treated by neoadjuvant, platin/5FU-based chemotherapy at the Department of Surgery at the Technische Universität München between 1991 and 2007 and were evaluated for response based on a standardized histopathological protocol [2–22]. Tumour regression was classified into 3 grades: tumour regression grade (TRG) 1, which consists of TRG1a (total tumour regression) and TRG1b (subtotal tumour regression: <10% residual tumour cells/tumour bed), TRG2 (partial tumour regression: 10–50% residual tumour cells/tumour bed) and TRG3 (minimal or no tumour regression: >50% residual tumour/tumour bed). Of the 480 patients, 121 patients demonstrated TRG2 and 63 of these were analysed in this study. The inclusion criterion was the availability of sufficient tumour tissue for the analysis of patients treated with at least 50% of the projected dose of chemotherapy. Patient characteristics and treatment protocols are shown in Table 1. To confirm the representative nature of the 63 analysed patients, the distribution of their clinicopathological parameters was compared to the 121-patient cohort and revealed no statistically significant differences.

Follow-up was calculated from the first day of treatment until the date of last contact with the patients. The median follow-up was 77.1 months (range: 28.5–108.5). The clinical endpoint of the study was overall survival (OS), which was defined as the time between the first day of chemotherapy and death by any cause. The median OS was 50.9 months (range: 4.5–108.5, 95% CI: 25.6–76.3), and 37 of the 63 patients died during follow up. This sample size and number of events are sufficiently large for a consistent estimation of all effect sizes investigated in this explorative study [23,24].

The comparison of gene expression between corresponding pre- and post-therapeutic tumour samples was performed for patients with TRG2 and TRG3 (each n = 22).

For the immunohistochemical analysis, pre- and post-therapeutic tumour samples from 21 patients with TRG1b, 21 patients with TRG2, 22 patients with TRG3 and of 16 patients treated by surgery alone were included. The selection criterion for these analyses was the availability of corresponding pre-therapeutic biopsies and post-therapeutic tumours.

### Ethics Statement

The study and the use of human tissues was approved by the local Institutional Review Board at the Technische Universität München (reference: 2158/08), and informed consent was obtained according to institutional regulations.

**Table 1.** Patient characteristics and treatment.

Variable	Category	n (%)
Patients		63 (100)
Age [yrs]	median	57.6
	range	35.0 – 73.0
Sex	female	16 (25)
	male	47 (74)
Tumour localisation	proximal	43 (68)
	medial	12 (19)
	distal	7 (11)
	total	1 (1)
Lauren classification	intestinal	24 (38)
	non-intestinal	39 (61)
Tumour grade	G1+2	5 (7)
	G3	58 (92)
Neoadjuvant chemotherapy	PLF <sup>1</sup>	48 (76)
	OLF <sup>2</sup>	3 (4)
	Epirubicin-PLF <sup>3</sup>	4 (6)
	Paclitaxel/Docetaxel-PLF <sup>4</sup>	8 (12)
Resection category	R0	53 (84)
	R1	10 (15)
ypT category <sup>5</sup>	ypT0-2	46 (73)
	ypT3+4	17 (27)
ypN category <sup>5</sup>	ypN0	24 (38)
	ypN1-3	39 (61)
ypM category <sup>5</sup>	ypM0	52 (82)
	ypM1	11 (17)

<sup>1</sup>Preoperative chemotherapy protocol: PLF: two cycles, each consisting of cisplatin (50 mg/m<sup>2</sup> body surface area (BSA)) at weeks 1, 3 and 5, and both leucovorin (500 mg/m<sup>2</sup> BSA) and 5-fluorouracil (2000 mg/m<sup>2</sup> BSA) at weeks 1, 2, 3, 4, 5 and 6 (PLF).

<sup>2</sup>Oxaliplatin (85 mg/m<sup>2</sup> BSA) replaces cisplatin in PLF.

<sup>3</sup>Additional epirubicin (30 mg/m<sup>2</sup> BSA) at weeks 2, 4, and 6,

<sup>4</sup>Additional paclitaxel (85 mg/m<sup>2</sup> BSA) or docetaxel (40–50 mg/m<sup>2</sup> BSA) at weeks 1, 3, and 5,

<sup>5</sup>TNM Classification of Malignant Tumors, 6<sup>th</sup> Edition, UICC.

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### RNA Extraction and Reverse Transcription

Total RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues after manual microdissection of tumour areas composed of at least 50% tumour cells. The RNA was purified by phenol and chloroform extraction and was reverse transcribed as described [25].

### Gene Expression Analysis

The 44 genes that were selected for analysis based on their potential role in CSC biology are included in Table 2. Gene expression was analysed by quantitative real time PCR (qRT-PCR) on custom-made TaqMan<sup>®</sup> low density arrays (Applied Biosystems Inc., Foster City, USA). Appropriate reference genes were determined by an analysis of ten candidate reference genes in 8 gastric carcinomas using the geNorm-algorithm [26]. *IPO8*, *POLR2A* and *UBC* were determined to be the most suitable reference genes and normalisation based on the geometric mean of these three genes was performed as described [26]. Reagents,

**Table 2.** Genes analysed by TaqMan® low density array.

Gene	Gene ID <sup>1</sup>	AssayID <sup>2</sup>	Amplicon-length [bp]
ABCB1	5243	Hs00184500_m1	67
ABCG2	9429	Hs01053790_m1	83
ALDH1A1	216	Hs00946916_m1	61
ASCL2	430	Hs00270888_s1	101
ATXN1	6310	Hs00165656_m1	97
AXIN1	8312	Hs00394718_m1	81
BMI1	648	Hs00180411_m1	105
CCND1	595	Hs00765553_m1	57
CD133	8842	Hs01009257_m1	80
CD24	100133941	Hs02379687_s1	140
CD34	947	Hs02576480_m1	63
CD44	960	Hs01075861_m1	70
CDH1	999	Hs01013953_m1	65
CDX2	1045	Hs01078080_m1	81
CHD1	1105	Hs00154405_m1	84
CTNNB1	1499	Hs00355045_m1	86
DKK3	27122	Hs00247426_m1	83
DNMT1	1786	Hs00154749_m1	77
DNMT3A	1788	Hs01027166_m1	79
DNMT3B	1789	Hs00171876_m1	55
FOXD3	27022	Hs00255287_s1	73
FZD1	8321	Hs00268943_s1	83
GADD45A	1647	Hs00169255_m1	123
GLI1	2735	Hs00171790_m1	80
GSK3B	2932	Hs00275656_m1	73
HDAC1	3065	Hs00606262_g1	149
HDAC2	3066	Hs00231032_m1	106
IHH	3549	Hs01081801_m1	103
KLF4	9314	Hs00358836_m1	110
LGR4	55366	Hs00173908_m1	68
LGR5	8549	Hs00173664_m1	112
LIN28	79727	Hs00702808_s1	143
MKI67	4288	Hs01032443_m1	66
MYC	4609	Hs00905030_m1	87
NANOG	79923	Hs02387400_g1	109
NOTCH1	4851	Hs01062014_m1	80
NOTCH2	4853	Hs01050719_m1	60
OLFM4	10562	Hs00197437_m1	85
POU5F1	5460	Hs00999632_g1	77
PTCH1	5727	Hs00970979_m1	63
SFRP1	6422	Hs00610060_m1	130
SHH	6469	Hs00179843_m1	70
SMO	6608	Hs01090242_m1	54
SOX2	6657	Hs01053049_s1	91
IPO8 <sup>3</sup>	10526	Hs00183533_m1	71
POLR2A <sup>3</sup>	5430	Hs00172187_m1	61
UBC <sup>3</sup>	7316	Hs00824723_m1	71

<sup>1</sup>www.ncbi.nlm.nih.gov/gene,<sup>2</sup>Applied Biosystems,<sup>3</sup>Reference genes.

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cycling conditions and software are included in the Supporting Information. Relative gene expression was quantified using the comparative  $\Delta\Delta C_t$  method [27].

### Immunohistochemistry

The monoclonal NOTCH2 antibody (C651.6DbHN) was obtained from the Developmental Studies Hybridoma Bank (DSHB, The University of Iowa, Department of Biology, Iowa City, USA). The staining procedure and examination of antibody specificity by Western blotting are described in the Supporting Information and Figure S1A.

Immunohistochemical staining was scored in a blinded fashion by two independent researchers (L.B. and R.L). Cytoplasmic and nuclear staining was evaluated separately. Negative, weak, medium or strong staining intensities were scored as 0, 1, 2 and 3, respectively. The percentage of tumour cells with stained cytoplasm/nucleus was scored as 0 (negative), 1 (<10%), 2 (10 to <50%), 3 (50 to <80%) and 4 ( $\geq$ 80%).

### Statistical Analysis

Conditional inference tests were used to determine the optimal cut-off-values of gene expression for the association with patient survival and to determine the p-values appropriate for maximally selected statistics [28]. Gene expression values above or equal to the optimal cut-off value were defined as high expression and gene expression values below the cut-off value were defined as low expression. Survival rates were estimated according to Kaplan-Meier curves and were compared by log-rank tests. Relative risks were estimated by determining hazard ratios (HRs) from Cox proportional hazard models. In the multivariate analysis, stepwise forward variable selection was performed based on likelihood ratio tests. The ratio of the number of variables in the model to the number of events was limited to 1:10 [24].

Comparisons of clinicopathological variables between groups were performed by Mann-Whitney-U tests. The  $\chi^2$ -test and Fisher's exact test were used for the comparison of relative frequencies where appropriate. Statistical differences of gene expressions between paired samples were analysed using the Wilcoxon signed rank test.

Unsupervised hierarchical cluster analysis was performed with Cluster 3.0 software [29] and Java TreeView software (version 1.1.5r2) [30]. Relative mRNA expression data were log-transformed, median-centred and normalised before applying complete linkage clustering with a distance matrix based on Pearson's correlation (uncentred).

A risk score was calculated by summarizing the products of the multiplication of the Cox regression coefficient of each gene in the model with the normalised gene expression values for each patient essentially as described [31] (details in Material and Methods S1 and Tables S4 and S6).

All statistical tests were two-sided and conducted in an explorative manner with a significance level of 0.05 using the SPSS 18.0 software (SPSS Inc., Chicago, IL 11.5) and R (R Foundation for Statistical Computing, Vienna, Austria).

The study complies with the reporting recommendations for tumour marker prognostic studies (REMARK criteria) [32].

### Analysis of Public Microarray Data

Publicly available gene expression array data of gastric carcinomas with descriptions of clinical characteristics and patient survival [33] were obtained from the BRB-Array Tools data archive ([http://linus.nci.nih.gov/~brb/DataArchive\\_New.html](http://linus.nci.nih.gov/~brb/DataArchive_New.html)) and analysed using BRB-Array Tools [34] (Supporting Information). Only advanced gastric carcinomas (T3/4) (n = 58) were

included in the analysis. The optimal cut-off values of *GSK3B*, *CTNNB1*, and *NOTCH2* expression were determined for an association with patient survival by the conditional inference tests for maximally selected statistics as described above.

## Results

### Gene Expression and Patient Survival

The gene expression profiling of the 63 tumours of patients with TRG2 showed, that high expression levels of *GSK3B*, *DNMT1* and *CTNNB1* were significantly associated with better survival (conditional inference test:  $p = 0.006$ ,  $0.041$ , and  $0.043$ , respectively). A moderate association with better survival was observed for high expression of *ABCG2* and *OLFM4* ( $p = 0.051$ ,  $p = 0.055$ ) and for low expression of *NOTCH2* ( $p = 0.071$ ) (Table 3). Univariate Cox-regression analysis demonstrated approximately concordant results (Table 4).

A multivariate Cox regression analysis including *GSK3B*, *CTNNB1*, *DNMT1* and the standard prognostic variables in GC, namely ypT, ypN, ypM and resection category revealed *GSK3B* as the second most important independent prognostic factor (HR:  $0.128$ , 95% CI:  $0.033$ – $0.492$ ,  $p = 0.003$ ) after distant metastasis (Table S1).

A cluster analysis encompassing all of the analysed genes revealed no patient groups that exhibited an association with OS. A cluster analysis of Wnt- and Notch signalling-associated genes produced the most significant association with OS when *GSK3B*, *CTNNB1* and *NOTCH2* were included ( $p = 0.002$ ) (Figure 1A and B). According to the results of the cluster analysis, we grouped the patients into three groups with different combinations of high or low expression of *GSK3B*, *CTNNB1* and *NOTCH2*, which was defined by the optimal cut-off-values for gene expression in association with patient survival. The group with *GSK3B*<sup>high</sup>, *CTNNB1*<sup>high</sup> and *NOTCH2*<sup>low</sup> expression showed the best survival, whereas the group with *GSK3B*<sup>low</sup>, *CTNNB1*<sup>low</sup> and *NOTCH2*<sup>high</sup> expression had the worst overall outcome ( $p < 0.001$ , Figure 1C).

Calculation of a risk score based on a multivariate Cox proportional hazard regression model of these three genes and dichotomisation of the patients according to the optimal cut-off value for OS into a high ( $n = 37$ ) and low ( $n = 26$ ) risk group demonstrated a statistically significant difference for OS (median survival of low and high risk patients: not reached and 37 months respectively;  $p < 0.001$ ). In addition, the difference in the respective survival rates at 1, 2, 3 and 5 years between the low and high risk group were statistically significant (Table S5).

**Table 3.** Gene expression and association with survival – conditional inference tests.

Gene	< cut-off		≥ cut-off		p-value
	n	median survival [mo]	n	median survival [mo]	
<i>GSK3B</i>	40	47.1	23	102.6	0.006
<i>DNMT1</i>	53	42.1	10	nr	0.041
<i>CTNNB1</i>	15	32.3	48	94.9	0.043
<i>ABCG2</i>	54	47.1	9	102.6	0.051
<i>OLFM4</i>	53	40.4	10	nr	0.055
<i>NOTCH2</i>	31	94.9	32	40.4	0.071

nr: median survival not reached.

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### Differential Gene Expression Analysis between Corresponding Pre- and Post-therapeutic Tumour Samples

We next determined, whether the expression levels of the genes that exhibited a significant or moderate association with OS ( $p < 0.1$ , Table 3 and 4) differ between pre-therapeutic biopsies and their corresponding post-therapeutic tumour specimen. Additionally, *POU5F1*, *LGR5* and *CCND1* were analysed, and tumour samples of patients with TRG2 or TRG3 (each  $n = 22$ ) were studied.

In patients with TRG2, the expression of *NOTCH2*, *POU5F1* and *LGR5* increased significantly between the pre- and the post-therapeutic specimens ( $p = 0.002$ ,  $0.028$  and  $0.017$ , respectively) and the expression of *DNMT1* decreased ( $p = 0.009$ ). In the group with TRG3, *POU5F1* exhibited a significant increase ( $p = 0.002$ ), while *DNMT1* and *CCND1* significantly decreased ( $p = 0.002$  and  $0.007$ , respectively).

Regarding the expression of the prognostic-relevant genes *GSK3B* and *CTNNB1*, no statistically significant differences were observed between the pre- and the post-therapeutic tumour samples (Table 5 and Tables S2 and S3).

### Analysis of Publically Available Array Data

To evaluate the general prognostic value of the *GSK3B*<sup>high</sup>, *CTNNB1*<sup>high</sup> and *NOTCH2*<sup>low</sup> expression pattern, we used a publically available expression array data set of gastric carcinomas [33]. The data set included the genes of interest and the relevant clinical information (OS, tumour stage) necessary to perform an analogous analysis. The determination of the optimal cut-off values of gene expression for correlation with survival and evaluation of the combined expression signature of *GSK3B*<sup>high</sup>, *CTNNB1*<sup>high</sup> and *NOTCH2*<sup>low</sup> identified a group of 10 patients who had a significantly longer OS ( $p = 0.017$ , median OS: not reached) compared with the 47 remaining patients (median OS: 14.0 mo, 95% CI:  $8.7$ – $19.4$ ) (Figure 1D).

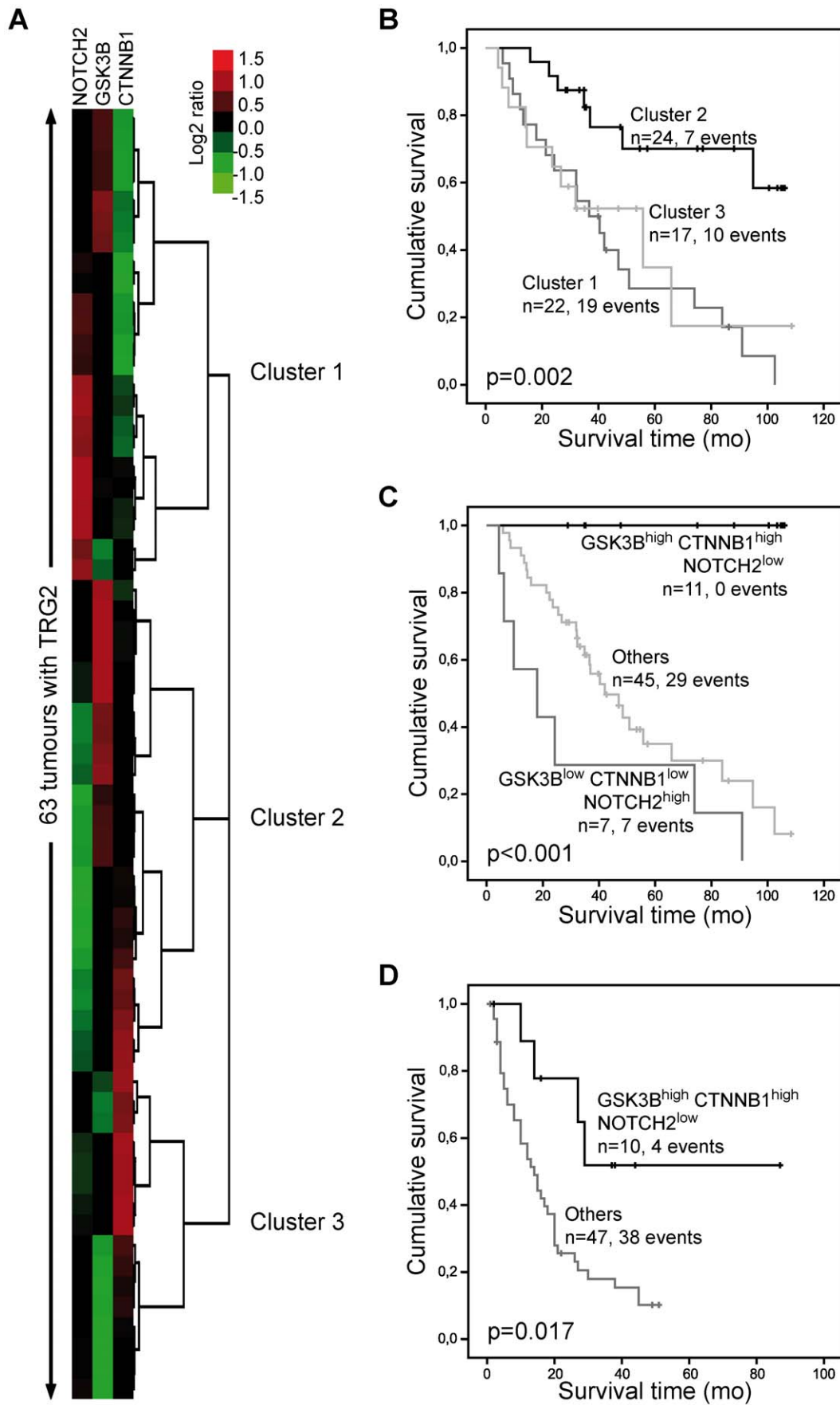
Calculation of the risk score for this patient group and dichotomisation of the patients according to the optimal cut-off value for OS showed a considerably longer OS for the low risk group ( $n = 27$ ) compared to the high risk group ( $n = 30$ ), although the difference was not statistically significant (median survival 21 versus 13 months,  $p = 0.110$ ). Considering the differences in the respective survival rates at 1, 2 and 3 years demonstrated analogous results with the most obvious difference at 2 years with a survival rate of 45% of the low risk patients and 21% of the high risk patients ( $p = 0.071$ ) (Table S7).

### Immunohistochemical Analysis of NOTCH2

To evaluate the differences in gene expression between the paired pre- and post-therapeutic tumours on the protein level, we performed immunohistochemistry and focused on NOTCH2 (Figure S1B and C). We analysed the same patient groups that had been studied on the mRNA level. In addition, 21 patients with TRG1b and a control group of 16 patients treated by surgery alone were included.

A comparison of cytoplasmic staining intensities between pre-therapeutic biopsies and their corresponding post-therapeutic tumours revealed a statistically significant increase in staining intensity in the post-therapeutic specimens from patients with TRG1b, 2 and 3 ( $p = 0.016$ ,  $0.001$ , and  $0.017$ , respectively). In contrast, no differences were observed in patients treated by surgery alone ( $p = 0.438$ ) (Figure 2). The percentage of stained cells was not significantly altered. Regarding nuclear staining, a significant decrease in staining intensity in the post-therapeutic





**Figure 1. Expression of *GSK3B*, *CTNNB1* and *NOTCH2* and association with survival. A)** Clustering of tumours based on expression of *GSK3B*, *CTNNB1* and *NOTCH2*. **B)** The Kaplan-Meier curves of the patient clusters show better survival of patients in cluster 2 (median OS not reached) compared to cluster 1 (median OS 36.7 mo, 95% CI 24.4–49.1) or cluster 3 (median OS 55.9 mo, 95% CI 16.7–95.0). **C)** Kaplan-Meier curves of patients based on the categorisation of tumours according to the optimal cut-off values for the three genes (*GSK3B*<sup>high</sup> *CTNNB1*<sup>high</sup> *NOTCH2*<sup>low</sup>: median OS not reached; *GSK3B*<sup>low</sup> *CTNNB1*<sup>low</sup> *NOTCH2*<sup>high</sup>: median OS 18.0 mo, 95% CI 0–39.5; Others: median OS 42.1 mo, 95% CI 28.3–55.9). **D)** Analysis of publically available array data of gastric cancer [32]. Kaplan-Meier curves of patients categorised according to the combined expression of *GSK3B*, *CTNNB1* and *NOTCH2* in the tumours using optimal cut-off values are shown (*GSK3B*<sup>high</sup> *CTNNB1*<sup>high</sup> *NOTCH2*<sup>low</sup>: median OS not reached; Others: median OS 14.6 mo, 95% CI 8.6–19.3). P-values were determined by the log-rank test.  
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tumour specimen was found in the group with TRG2 ( $p = 0.007$ ), TRG3 ( $p = 0.015$ ) and in the control group not treated by chemotherapy ( $p = 0.016$ ). A significant decrease in the percentage of cells with stained nuclei was observed in the group with TRG1b ( $p = 0.005$ ), TRG2 ( $p < 0.001$ ), TRG3 ( $p = 0.003$ ) as well as in the control group ( $p = 0.001$ ).

## Discussion

Our study analysing the expression of CSC related genes in residual gastric cancer cells after neoadjuvant chemotherapy identified a gene signature with a high prognostic impact composed of *GSK3B*, the  $\beta$ -catenin gene *CTNNB1* and *NOTCH2*. Interestingly, high expression levels of *CTNNB1* and *GSK3B* were associated with increased survival.  $\beta$ -catenin is a key molecule in the transmission of Wnt signalling to the nucleus and drives multiple cellular processes [35]. Aberrant Wnt signalling has been demonstrated in up to 46% of GCs, and both Wnt/ $\beta$ -catenin and proliferation/stem cell expression signatures indicating the respective pathway activation, were associated with decreased patient survival [36]. *CTNNB1* mRNA levels can modulate Wnt signalling [37], and Wnt activity has been linked to cancer stemness in the colon [38]. Given these data, a negative association of *CTNNB1* expression in residual tumour cells after chemotherapy and the survival of the patients would have been expected. However,  $\beta$ -catenin is also part of the E-cadherin-catenin complex and alterations in this cell adhesion complex have been associated with worse prognosis in various tumours [39]. In GC, conflicting results regarding the association between  $\beta$ -catenin expression and patient prognosis exist [40,41].

It is important to note, that *GSK3B* is also a multifunctional protein involved in various signalling networks and antagonises Wnt signalling by mediating the degradation of  $\beta$ -catenin, although a Wnt-activating function has also been described [42–44]. Increased expression of *GSK3B* was associated with a favourable prognosis in our study, which is consistent with a report of higher *GSK3B* mRNA expression associated with better survival in ovarian high-grade serous carcinomas [45].

**Table 4. Gene expression and association with survival – univariate Cox regression analysis.**

Gene	HR <sup>1</sup>	95% CI <sup>2</sup>	p-value
<i>GSK3B</i>	0.220	0.064–0.756	0.016
<i>DNMT1</i>	0.379	0.138–1.042	0.060
<i>CTNNB1</i>	0.567	0.305–1.052	0.072
<i>ABCG2</i>	0.851	0.707–1.024	0.087
<i>OLFM4</i>	0.987	0.969–1.006	0.191
<i>NOTCH2</i>	3.326	0.935–11.840	0.064

<sup>1</sup>hazard ratio,

<sup>2</sup>95% confidence interval.

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Higher *NOTCH2* expression levels correlated with worse survival in our GC patients. The Notch receptor family encompasses four members, and *NOTCH1* and *NOTCH2* have been implicated to enhance gastric cancer progression [46,47]. Furthermore, an association of *NOTCH1* expression with poor prognosis has been reported [48], which is not consistent with our findings; however, due to the differences in the study populations, these results are not directly comparable.

Given the highly significant association of the gene signature encompassing *CTNNB1*, *GSK3B* and *NOTCH2* with patient survival and the significant risk score–based classification of the patients into a high and low risk group, we were particularly interested, if this were related to chemotherapy. The comparison of *GSK3B* and *CTNNB1* expression levels between pre- and post-therapeutic tumour samples revealed no clear differences, whereas a significant increase in the expression of *NOTCH2* was found. This finding suggests that *CTNNB1* and *GSK3B* expression may reflect a property of the primary tumour that is not altered by chemotherapy and that *NOTCH2* expression in the residual tumour cells is at least partly related to this treatment.

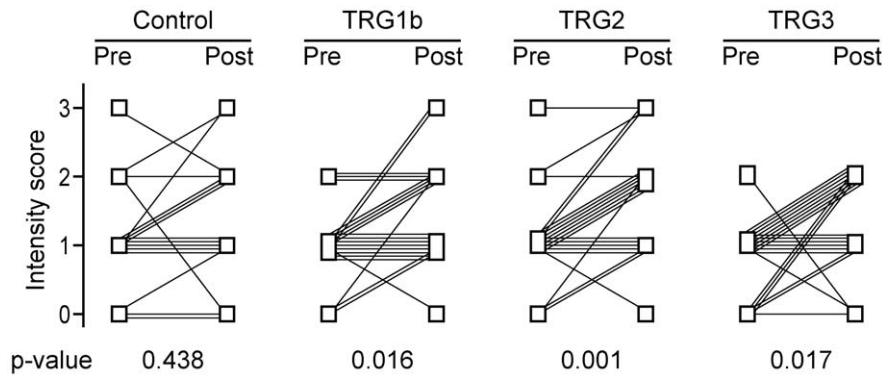
An analysis of the prognostic significance of the three-genes in publically available genome-wide expression data of advanced GC demonstrated a significant association for the specific gene expression pattern of *GSK3B*<sup>high</sup>, *CTNNB1*<sup>high</sup> and *NOTCH2*<sup>low</sup> expression and increased patient survival and a similar tendency considering the risk score-based classification.

**Table 5. Alterations of expression between pre- and post-therapeutic tumours of patients with tumour regression grade (TRG) 2 and 3.**

Alteration	TRG2		TRG3	
	Gene	p-value <sup>1</sup>	Gene	p-value <sup>1</sup>
<b>Increase</b>				
	<i>NOTCH2</i>	0.002	<i>NOTCH2</i>	0.062
	<i>POU5F1</i>	0.028	<i>POU5F1</i>	0.002
	<i>LGR5</i>	0.017		
	<i>CTNNB1</i>	0.062		
<b>No change</b>				
	<i>ABCG2</i>	0.263	<i>LGR5</i>	0.249
	<i>GSK3B</i>	0.263	<i>CTNNB1</i>	0.733
	<i>OLFM4</i>	0.211	<i>ABCG2</i>	0.485
	<i>CCND1</i>	0.178	<i>GSK3B</i>	0.709
			<i>OLFM4</i>	0.961
<b>Decrease</b>				
	<i>DNMT1</i>	0.009	<i>CCND1</i>	0.007
			<i>DNMT1</i>	0.002

<sup>1</sup>Wilcoxon signed rank test.

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**Figure 2. Alterations in the immunohistochemical staining for NOTCH2 between pre-therapeutic biopsies and their corresponding post-therapeutic tumours.** Alterations of cytoplasmic staining intensities are shown. Each line indicates the alteration of the immunohistochemical staining score between the pre-therapeutic biopsy (Pre) and the corresponding post-therapeutic tumour specimen (Post) for each case. P-values were determined by the Wilcoxon signed rank test (exact). doi:10.1371/journal.pone.0044566.g002

This finding supports the interpretation that the prognostic effect observed in our study mainly reflects a property of the primary gastric tumour, suggesting a critical role for these genes in the biology of these tumours. In addition, the prognostic effect observed might be enhanced by an increase in the expression of *NOTCH2* in the residual tumour after chemotherapy. Thus, if validated in a prospective study, this three gene signature might be useful for risk stratification of GC patients and additionally may guide postoperative treatment after neoadjuvant chemotherapy.

Our results are reminiscent of a recent study analysing a CSC-derived gene signature that predicts tumour recurrence in the colon and demonstrates that the elevated expression of Wnt target genes is indicative of a favourable prognosis [48]. The authors provide evidence that this association more likely reflects the differentiation status of the malignant tissue rather than the number of CSCs [49].

In considering the alterations of *NOTCH2* expression between corresponding pre- and post-therapeutic tumours, it is important to note that comparing gene expression at the mRNA-level, cannot distinguish whether these alterations reflect a relative enrichment of the cells expressing this gene, whether they are due to the chemotherapeutic agents affecting gene transcription in the cells *per se*, or whether the alterations reflect mere sampling differences. To clarify this issue we analysed *NOTCH2* protein expression by immunohistochemistry and included tumours from patients treated by surgery alone. Based on the cytoplasmic staining, our results confirm an increase in *NOTCH2* expression at the protein level in the post-therapeutic tumours and they demonstrate that the observed differences are likely to be restricted to patients treated by chemotherapy. Of note, the increase in *NOTCH2* expression was related to an increase in the cytoplasmic staining intensity rather than to an increase in the number of cells expressing *NOTCH2*. This result argues against an enrichment of a subpopulation of *NOTCH2*-expressing tumour cells and more likely suggests a chemotherapy-induced increase in gene expression in the tumour cells, which may be related to the tumour biological features after neoadjuvant treatment. However, a clear distinction between these possibilities may be limited by the semiquantitative evaluation of immunohistochemical staining. As similar alterations in nuclear staining were observed in all tumour groups including the control we considered these changes as unrelated to chemotherapy.

Irrespective of the mechanism and the true nature of the residual tumour cells expressing *NOTCH2*, our results may have

therapeutic implications. Notch signalling has emerged as a potential new therapeutic target, and gamma-secretase inhibitors, which inhibit the processing of the Notch receptors, are currently being evaluated in clinical trials [50]. Our study suggests that targeting Notch signalling may also represent a new strategy to treat GC patients. As an adverse prognostic effect was only associated with *NOTCH2* and not *NOTCH1*, our data also indicate that a detailed characterisation of the individual Notch receptors and a thorough functional investigation are mandatory and further strongly favour the development of Notch paralog-specific inhibitory agents.

A significant increase in *POU5F1* expression was observed after chemotherapy in the resected specimens in our study. The *POU5F1* transcription factor is essential for the maintenance of self-renewal, and its high expression in residual cancer cells after radiochemotherapy is correlated with poor prognosis in colon cancer [51]. Interestingly we also observed an increased expression of *LGR5*, a promising intestinal CSC marker, after chemotherapy in tumours with TRG2 [18]. These results are compatible with the potential enrichment of drug-resistant tumour cells expressing *POU5F1* or *LGR5*, but the underlying mechanism for these alterations and the particular properties of the cells expressing these genes remain to be determined.

In our study, no association with survival were observed for the cell surface molecules CD44 or CD133, both of which have been widely used to identify putative CSCs in various tumours [4–11]. This result supports recent findings demonstrating that these cell surface molecules do not identify CSCs in primary gastric tumours [20].

Taken together, our findings demonstrate that the expression signature of *GSK3B*<sup>high</sup>, *CTNNB1*<sup>high</sup> and *NOTCH2*<sup>low</sup> in chemotherapy-resistant residual GC tumour cells is a strong predictor for favourable patient prognosis. This prognostic relevance was also demonstrated for GC patients using publicly available gene expression data. The results of the differential expression analysis of the pre- and post-therapeutic tumour specimen also suggests that the impact of *GSK3B* and *CTNNB1* to this signature is not dependent on chemotherapy but rather related to a property of the primary tumour. They further indicate a prominent role for *NOTCH2* and chemotherapy resistance in GC, which is more likely related to an effect of the chemotherapeutic agents on *NOTCH2* expression rather than to an enrichment of *NOTCH2* expressing tumour cells.

## Supporting Information

**Figure S1** Western blot and immunohistochemistry with the anti-NOTCH2 antibody. **A)** The antibody directed against the NOTCH2 intracellular domain specifically detects the full length NOTCH2 protein above the 250 kDa marker as well as the cleaved forms NOTCH Extracellular Truncated (NEXT) and NOTCH Intracellular Domain (NICD) at approximately 110 kDa. **B)** A weak cytoplasmic immunohistochemical staining in the pre-therapeutic biopsy sample and **C)** a strong cytoplasmic staining in the corresponding post-therapeutic tumour with TRG2 is shown. Scale bars indicate 50  $\mu$ m. (TIF)

**Table S1** Multivariate Cox regression analysis. Gene expression of *GSK3B*, *CTNNT1*, *DNMT1* and the standard prognostic variables in GC, ypT, ypN, ypM and resection category were included in the model. (DOC)

**Table S2** Gene expression data of the pre- and corresponding post-therapeutic tumour samples of patients with TRG2. (DOC)

**Table S3** Gene expression data of the pre- and corresponding post-therapeutic tumour samples of patients with TRG3. (DOC)

**Table S4** Multivariate Cox regression data for the own dataset. (DOC)

**Table S5** Relative survival rates based on the dichotomised risk score (own data). (DOC)

**Table S6** Multivariate Cox regression data for the publically available dataset. (DOC)

**Table S7** Relative survival rates based on the dichotomised risk score (public data). (DOC)

**Material and Methods S1** Information on experimental details on quantitative real time PCR, immunohistochemistry, Western blotting, the analysis of public microarray data and multivariate Cox regression based risk scores. (DOC)

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## Author Contributions

Conceived and designed the experiments: LB GK. Performed the experiments: LB. Analyzed the data: LB RL. Contributed reagents/materials/analysis tools: KB KO AN HH. Wrote the paper: LB GK. Statistical analysis: AH.

## References

- Lordick F, Siewert JR (2005) Recent advances in multimodal treatment for gastric cancer: a review. *Gastric Cancer* 8: 78–85.
- Becker K, Langer R, Reim D, Novotny A, Meyer zum Buschenfelde C, et al. (2011) Significance of histopathological tumor regression after neoadjuvant chemotherapy in gastric adenocarcinomas: a summary of 480 cases. *Ann Surg* 253: 934–939.
- Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. *Nature* 414: 105–111.
- O'Brien CA, Kreso A, Jamieson CH (2010) Cancer stem cells and self-renewal. *Clin Cancer Res* 16: 3113–3120.
- Clevers H (2011) The cancer stem cell: premises, promises and challenges. *Nat Med* 17: 313–319.
- Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, et al. (2008) Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst* 100: 672–679.
- Lee HE, Kim JH, Kim YJ, Choi SY, Kim SW, et al. (2011) An increase in cancer stem cell population after primary systemic therapy is a poor prognostic factor in breast cancer. *Br J Cancer* 104: 1730–1738.
- Shats I, Gatz ML, Chang JT, Mori S, Wang J, et al. (2011) Using a stem cell-based signature to guide therapeutic selection in cancer. *Cancer Res* 71: 1772–1780.
- Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, et al. (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 40: 499–507.
- Wang T, Ong CW, Shi J, Srivastava S, Yan B, et al. (2011) Sequential expression of putative stem cell markers in gastric carcinogenesis. *British Journal of Cancer* 105: 658–665.
- Wakamatsu Y, Sakamoto N, Oo HZ, Naito Y, Uraoka N, et al. (2012) Expression of cancer stem cell markers ALDH1, CD44 and CD133 in primary tumor and lymph node metastasis of gastric cancer. *Pathology International* 62: 112–119.
- Takebe N, Ivy SP (2010) Controversies in cancer stem cells: targeting embryonic signaling pathways. *Clin Cancer Res* 16: 3106–3112.
- Takaishi S, Okumura T, Wang TC (2008) Gastric cancer stem cells. *J Clin Oncol* 26: 2876–2882.
- Vries RG, Huch M, Clevers H (2010) Stem cells and cancer of the stomach and intestine. *Mol Oncol* 4: 373–384.
- Saikawa Y, Fukuda K, Takahashi T, Nakamura R, Takeuchi H, et al. (2010) Gastric carcinogenesis and the cancer stem cell hypothesis. *Gastric Cancer* 13: 11–24.
- Qiao XT, Gumucio DL (2011) Current molecular markers for gastric progenitor cells and gastric cancer stem cells. *J Gastroenterol* 46: 855–865.
- Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, et al. (2004) Gastric cancer originating from bone marrow-derived cells. *Science* 306: 1568–1571.
- Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, et al. (2010) Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 6: 25–36.
- Zhang C, Li C, He F, Cai Y, Yang H (2011) Identification of CD44+CD24+ gastric cancer stem cells. *J Cancer Res Clin Oncol* 137: 1679–1686.
- Rocco A, Liguori E, Pirozzi G, Tirino V, Compare D, et al. (2012) CD133 and CD44 cell surface markers do not identify cancer stem cells in primary human gastric tumours. *J Cell Physiol* 127: 2686–2693.
- Schuhmacher CP, Fink U, Becker K, Busch R, Dittler HJ, et al. (2001) Neoadjuvant therapy for patients with locally advanced gastric carcinoma with etoposide, doxorubicin, and cisplatin. Closing results after 5 years of follow-up. *Cancer* 91: 918–927.
- Ott K, Sendler A, Becker K, Dittler HJ, Helmberger H, et al. (2003) Neoadjuvant chemotherapy with cisplatin, 5-FU, and leucovorin (PLF) in locally advanced gastric cancer: a prospective phase II study. *Gastric Cancer* 6: 159–167.
- Vittinghoff E, McCulloch CE (2007) Relaxing the rule of ten events per variable in logistic and Cox regression. *Am J Epidemiol* 165: 710–718.
- Peduzzi P, Concato J, Feinstein AR, Holford TR (1995) Importance of events per independent variable in proportional hazards regression analysis. II. Accuracy and precision of regression estimates. *J Clin Epidemiol* 48: 1503–1510.
- Napieralski R, Ott K, Kremer M, Specht K, Vogelsang H, et al. (2005) Combined GADD45A and thymidine phosphorylase expression levels predict response and survival of neoadjuvant-treated gastric cancer patients. *Clin Cancer Res* 11: 3025–3031.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034.
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3: 1101–1108.
- Hothorn T, Zeileis A (2008) Generalized maximally selected statistics. *Biometrics* 64: 1263–1269.
- de Hoon MJ, Imoto S, Nolan J, Miyano S (2004) Open source clustering software. *Bioinformatics* 20: 1453–1454.
- Saldanha AJ (2004) Java Treeview—extensible visualization of microarray data. *Bioinformatics* 20: 3246–3248.
- Cho JY, Lim JY, Cheong JH, Park YY, Yoon SL, et al. (2011) Gene expression signature-based prognostic risk score in gastric cancer. *Clin Cancer Res* 17: 1850–1857.
- McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, et al. (2005) Reporting recommendations for tumour MARKer prognostic studies (REMARK). *Br J Cancer* 93: 387–391.
- Chen X, Leung SY, Yuen ST, Chu KM, Ji J, et al. (2003) Variation in gene expression patterns in human gastric cancers. *Mol Biol Cell* 14: 3208–3215.



34. Zhao Y, Simon R (2008) BRB-ArrayTools Data Archive for human cancer gene expression: a unique and efficient data sharing resource. *Cancer Inform* 6: 9–15.
35. MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17: 9–26.
36. Ooi CH, Ivanova T, Wu J, Lee M, Tan IB, et al. (2009) Oncogenic pathway combinations predict clinical prognosis in gastric cancer. *PLoS Genet* 5: e1000676.
37. Freeman TJ, Smith JJ, Chen X, Washington MK, Roland JT, et al. (2012) Smad4-mediated signaling inhibits intestinal neoplasia by inhibiting expression of beta-catenin. *Gastroenterology* 142: 562–571 e562.
38. Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, et al. (2010) Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 12: 468–476.
39. Filiz AI, Senol Z, Sucullu I, Kurt Y, Demirbas S, et al. (2010) The survival effect of E-cadherin and catenins in colorectal carcinomas. *Colorectal Dis* 12: 1223–1230.
40. Nabais S, Machado JC, Lopes C, Seruca R, Carneiro F, et al. (2003) Patterns of beta-catenin expression in gastric carcinoma: clinicopathological relevance and mutation analysis. *Int J Surg Pathol* 11: 1–9.
41. Grabsch H, Takeno S, Noguchi T, Hommel G, Gabbert HE, et al. (2001) Different patterns of beta-catenin expression in gastric carcinomas: relationship with clinicopathological parameters and prognostic outcome. *Histopathology* 39: 141–149.
42. Zeng X, Tamai K, Doble B, Li S, Huang H, et al. (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* 438: 873–877.
43. Luo J (2009) Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. *Cancer Lett* 273: 194–200.
44. Espinosa L, Ingles-Esteve J, Aguilera C, Bigas A (2003) Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. *J Biol Chem* 278: 32227–32235.
45. Espinosa I, Catus L, Canet B, D'Angelo E, Munoz J, et al. (2011) Gene expression analysis identifies two groups of ovarian high-grade serous carcinomas with different prognosis. *Mod Pathol* 24: 846–854.
46. Yeh TS, Wu CW, Hsu KW, Liao WJ, Yang MC, et al. (2009) The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. *Cancer Res* 69: 5039–5048.
47. Tseng YC, Tsai YH, Tseng MJ, Hsu KW, Yang MC, et al. (2011) Notch2-induced COX-2 expression enhancing gastric cancer progression. *Mol Carcinog*.
48. Li DW, Wu Q, Peng ZH, Yang ZR, Wang Y (2007) [Expression and significance of Notch1 and PTEN in gastric cancer]. *Ai Zheng* 26: 1183–1187.
49. de Sousa EMF, Colak S, Buikhuisen J, Koster J, Cameron K, et al. (2011) Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. *Cell Stem Cell* 9: 476–485.
50. Groth C, Fortini ME (2012) Therapeutic approaches to modulating Notch signaling: Current challenges and future prospects. *Semin Cell Dev Biol*.
51. Saigusa S, Tanaka K, Toiyama Y, Yokoe T, Okugawa Y, et al. (2009) Correlation of CD133, OCT4, and SOX2 in rectal cancer and their association with distant recurrence after chemoradiotherapy. *Ann Surg Oncol* 16: 3488–3498.